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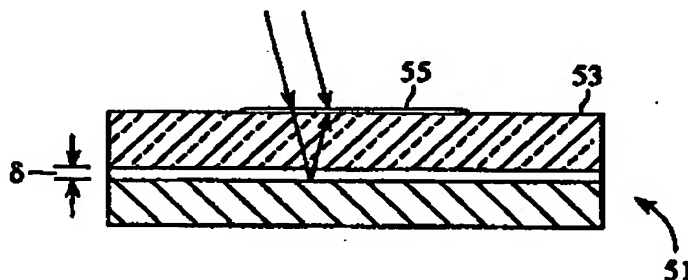## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: OPTICAL SUBSTRATE FOR ENHANCED DETECTABILITY OF FLUORESCENCE

(57) Abstract

A sample substrate (51, Fig. 4A) for use in a fluorescence imaging system (Fig. 1) includes a rigid base with a specularly reflective surface, typically metal, on which is deposited a transparent coating layer (53). The coating layer has a thickness selected so that a particular fluorescence excitation wavelength, corresponding to a specified fluorescent constituent to be sought in sample material, has an optical path from the top of the coating layer to the reflecting surface in the base of substantially an odd multiple of one-quarter wavelength, so that the standing wave of the fluorescence excitation wavelength of light incident of the substrate has an antinode (61) located at or near where sample material would be disposed on top of the coating layer. This maximizes fluorescence excitation of the sample on the reflective substrate. The transparent coating layer may be a dielectric material (e.g. silica) or may be a multilayer structure with a top layer of biologically active material for binding a specified sample constituent.

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Description

OPTICAL SUBSTRATE FOR
ENHANCED DETECTABILITY OF FLUORESCENCE

5

TECHNICAL FIELD

The present invention relates to sample substrates, such as plates, slides and cells, for use in examining, indicating, analyzing or identifying fluores-

10   cent, phosphorescent or luminescent sample materials, e.g. tagged molecular biological specimens, and in particular relates to such sample holders whose optical structures are adapted for enhancing fluorescence detection and imaging.

15

BACKGROUND ART

Fluorescence microscopy is often used in the fields of molecular biology, biochemistry and other life sciences for analyzing biological molecules, including

20   nucleic acids (DNA, RNA) and proteins (enzymes, antigens, etc.) that have been tagged or labeled with fluorescent probes. One such use is DNA diagnostics, such as for gene detection, in which a DNA sample is deposited on and bound to a glass substrate by means of a chemical binding

25   agent, such as an aminosilane, present on the substrate surface, and a reagent, such as a carbodiimide vapor. The bound DNA on the substrate can then be imaged by fluorescence. The fluorescence of a sample was originally assessed by visual inspection through a conventional

30   microscope, but this manual method has proved time-consuming and costly. Many different high-speed automated fluorescence imaging systems are now available.

An important figure of merit for fluorescence detection and measurement instruments is sensitivity,

35   which is primarily determined by the signal-to-noise ratio (SNR) of the optical imaging system of the instrument. A well-designed imaging system has a signal-to-noise ratio that is limited by its light collection

ability and not by internal noise sources. The
theoretical SNR of such a system is expressed in terms of
the number of photoelectrons at the cathode when using a
photomultiplier tube (PMT), which in turn essentially
5   depends upon the number of photons that reach the
detector from the area of interest on the sample
substrate, the quantum efficiency of the detector, and
the number of dark electrons generated by the detector.

$$SNR = S / [S + 2B]^{1/2} \quad ,$$

10   where B is the total background noise and S is the
measured signal less B. One obvious approach to
increasing SNR, and thereby improving sensitivity, is to
reduce background noise. Sources of background noise
include specular or diffuse reflection of the
15   fluorescence-stimulating laser light from the sample,
autofluorescence of the substrate holding the sample,
autofluorescence from the optics in the light path of the
optical imaging system, stray light, and dark current of
the detector. Stray light reaching the detector can be
20   significantly reduced by proper size and placement of
apertures in the imaging system. Both stray light and
much of the reflected laser light can be rejected, while
passing the fluorescent light, by using dichroic and
other spectral filters and beamsplitters in the system.
25   Autofluorescence of the optical elements can be reduced
by avoiding use of lens cements in the light path, using
glass instead of polymeric lenses, or using curved
mirrors instead of lenses wherever possible. Autofluo-
rescence of the substrate can be reduced by using low
30   fluorescence materials, such as an ultrathin or opaque
glass substrate. For example, in U.S. Pat. No. 5,095,213
Strongin discloses a plastic slide that is rendered
opaque and substantially nonfluorescent with a quantity
of black carbon powder in the plastic. Another way of
35   handling autofluorescence is to use a pulsed or modulated
excitation and to take advantage of the differences in
emission decay rates between background fluorescence and
specimen fluorescence, as disclosed in U.S. Pat. Nos.

4,877,965 to Dandiker et al. and 5,091,653 to Creager et
al.

In U.S. Pat. No. 5,552,272, Bogart discloses an
assay system and method for detecting the presence or
5   amount of an analyte of interest. It includes a test
substrate with an optically active surface that enhances
the color contrast, i.e. differences in the observed
wavelength (or combination of wavelengths) of light from
the surface, between the presence and absence of the
10   analyte in a sample applied onto the test substrate. In
particular, the substrate may comprise a reflective solid
optical support, such as a silicon wafer or metallic
(e.g., aluminum) base, with an optical thin film coating
thereon. The coating may comprise several layers,
15   including for example an attachment layer on the upper
surface of the support, and a receptive layer on the
upper surface of the attachment layer containing a
specific binding partner for the analyte of interest.
The total coating thickness is selected to cause incident
20   light to undergo thin film interference upon reflection,
such that a specific color is produced. Specifically,
the coating material(s) should have an overall thickness
of a quarterwave of the unwanted color to be attenuated
so that destructive interference of that color will
25   occur. The substrate therefore has a particular back-
ground color, which can then be used as a comparative
reference against a different observed color when an
analyte of interest is present. Both qualitative visual
inspection and quantitative instrumented measurement are
30   suggested. Polarization contrast by means of an ellip-
someter is also suggested.

One example of the use to which the Bogart
invention has been put by Biostar, Inc. of Boulder,
Colorado, the assignee of the aforementioned patent, is
35   an optical immunoassay (OIA) diagnostic screening test
for the rapid detection (in under 30 minutes) of the
presence of specific antigens of infectious pathogens n a
sample taken from a patient. Commercial products include

test kits for group A and group B streptococci and for
chlamydia trachomatis. These particular assays are given
as examples in the Bogart patent, are described in
package inserts for the corresponding Biostar products
5   and are also described in a number of published articles
in medical journals. Briefly, they all rely on direct
visual detection of a change in the color of light
reflection off of the test substrate due to a physical
change in the optical thickness of a molecular thin film
10   coating on the substrate surface which results from
binding reactions between an immobilized antibody on the
test surface and a specific antigen that may be present
in a drop of sample liquid applied to the test surface.
The original bare test surface has a thin film thickness
15   that results in a predominant visual background gold
color when white light is reflected off of the surface.
The antigen-antibody binding reaction that occurs when
the specific antigen of interest is present in the
applied sample results in an increase in the thin film
20   thickness that causes a corresponding change in the color
of the test surface from gold to purple. If on the other
hand, the antigen is not present in the sample, no
binding takes place, the original thin film thickness
remains unchanged and the test surface retains its
25   original gold color, indicating a negative result. This
diagnostic assay tool is very sensitive and easily
interpreted.

          Bogart further discloses, in another embodiment
of his invention (Fig. 17 of the aforementioned patent),
30   the use of these substrates for enhanced fluorescence
detection. After the analyte of interest has been bound
to the surface by reaction with the specific binding
partner in the receptive layer of the substrate coating,
fluorescent label molecules may be attached to the
35   analyte. In particular, the fluorescent molecules may be
attached to any suitably selective and specific receptive
material or reagent, such as a secondary antibody, and
applied to the surface. The fluorescent labels are thus

bound to the analyte of interest on the surface, if
present, and immobilized to the surface through the
analyte bridge. Directing light of an excitation
wavelength onto the surface stimulates fluorescence of
5   any of the label bound to the surface, thereby revealing
the presence of the analyte of interest. Because the
maximum fluorescence wavelength may not be shifted far
enough from the excitation wavelength to be
distinguished, the reflective substrate may have an
10   antireflection layer whose thickness is selected to
suppress reflection of the excitation wavelength, thereby
reducing the background noise reaching the detector.
Bogart states that the fluorescent signal generation is
not dependent on the film thickness.
15   Even if the background noise is minimized (and
even if the substrate is constructed so that the
reflected or fluorescent sample signal stands out more
clearly against the substrate's background by way of
contrast), the maximum possible signal-to-noise ratio
20   (SNR_{max}) is still limited to: $SNR_{max} = S^{1/2}$. Though the
fluorescence signal S might be increased by increasing
the output power of the laser, reflected laser noise will
also increase, with possibly little improvement in the
resulting SNR.
25   An object of the present invention is to
provide an improved sample substrate which provides
increased sample excitation and fluorescence emission.

DISCLOSURE OF THE INVENTION
30   The object has been met with a reflective
sample substrate having a transparent coating layer
thereon with controlled thickness that has been selected
to ensure that a molecular sample placed on top of the
coating layer is located at an antinode for the excita-
35   tion light. In particular, the substrate includes a
rigid base with a specularly reflective upper surface.
The transparent coating on the upper surface of the base
has a thickness selected such that for a particular

excitation wavelength of light at normal incidence, the
optical path from the top of the coating to the base
reflecting surface is substantially an odd multiple (1,
3, 5, etc.) of one-quarter wavelength of the excitation

5   light. The optical path length of the material is
defined by the wavelength of light, the index of
refraction of the material, and the angle of propagation
through the material. Note also that the reflecting
surface of the base is actually slightly below its

10   physical surface by an amount equal to the sum of the
skin (or penetration) depth of the reflective surface
material and the optical depth of any surface oxidation
on the base. A narrow excitation frequency linewidth is
preferred.

15   The base can be made completely of metal or may
be composed of a rigid bottom layer with a top metal
coating. The metal can be aluminum, silver, gold or
rhodium. The transparent coating may be a single layer
of dielectric material, such as silica, alumina or a

20   fluoride material (such as MgF_2). Alternatively, the
transparent coating could be a multilayer coating with
the top layer being a chemically reactive material for
binding a specified biological sample constituent
thereto.

25   By placing the sample on the coating layer at
or near the antinode of the excitation light, maximum
fluorescence excitation occurs. A reflective substrate
also enhances fluorescence collection by nearly doubling
the solid collection angle of a fluorescence imaging

30   microscope system. Thus, the total fluorescence signal
is increased, leading to a much improved signal-to-noise
ratio. Also, because the coating layer is very thin,
there is reduced fluorescence background noise from this
material.

35

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic side plan of a fluorescence microscope system for use with the substrate of the present invention.

5   Fig. 2 is a general perspective view of a sample substrate of the present invention being used with a fluorescence imaging system like the microscope of Fig. 1.

Figs. 3A and 4A are close-up side section views
10   of two sample substrates, one without a transparent coating (Fig. 3A) and the other, in accord with the present invention, having a transparent coating layer (Fig. 4A).

Figs. 3B and 4B are graphs of electric field
15   strength |E| versus depth for the substrates of Figs. 3A and 4A, respectively, illustrating the advantage of the inventive substrate of Fig. 4A.

BEST MODE OF CARRYING OUT THE INVENTION
20   The sample substrate of the present invention can be used in any of a wide number of possible fluorescence microscope systems, including, for example, those described in U.S. Patents 4,284,897 to Sawamura et al., 5,091,652 to Mathies et al., 5,296,700 to Kumagai,
25   5,381,224 to Dixon et al., and 5,504,336 to Noguchi, as well as U.S. patent applications 08/595,355, 08/616,174 and 08/791,684, all assigned to the assignee of the present invention. One preferred fluorescence imaging system for use with the present invention is illustrated
30   in Fig. 1.

A light source 18, such as a laser, produces a light beam 19. The beam is preferably a collimated beam of monochromatic coherent light. However, a noncoherent source, such as a light emitting diode (LED) could be
35   used and a noncollimated source could be coupled to collimating optics to create a collimated beam. If the beam 19 is not monochromatic, it may be directed through a filter 20 to reduce any unwanted wavelengths.

The beam 19 is then directed through a beam
expander 21 and reflected by a beamsplitter 23 onto a
scanning device 25. Any scanning mechanism that produces
a two-dimensional scan may be used. For example, the
5   scanner 25 may have a first beam reflecting element 43,
such as a galvanometer mirror or rotating polygon,
pivotable or rotatable about a first axis A and moved by
a motor 45, and a movable platform or turntable 47
rotated by a stepper motor 49 about a second axis B that
10   is orthogonal to the first axis A, and upon which the
first reflecting element 43 is supported. Reflector 43
need not have a planar reflecting surface, but could be
concave or convex in one or both axes, or even have a
diffracting surface.
15   The scanning beam is directed through an
objective lens 27 to illuminate a spot, line or area on a
sample 29. The objective 27 is preferably telecentric
(or near telecentric) in the image plane so that the
chief ray of the beam is always incident at substantially
20   a right angle to the sample surface regardless of the
scan position. With respect to the incident beam, the
objective's focal plane should be proximate to the sample
29. The objective 27 provides coaxial illumination and
collection. To maximize collection efficiency, it is
25   preferred that the objective lens 27 have a large
numerical aperture. The objective 27 preferably has an
external pupil that coincides with the scan axes A and B
at the reflective surface 43 so that collected light is
directed as a retro-beam back toward the beamsplitter 23.
30   The illuminating light beam 19 is an excitation beam that
stimulates fluorescent light emission from the sample 29
at the illuminated spot. The fluorescent light is then
collected by the objective lens 27, acting as a
condenser, and directed as a retro-beam back along the
35   incident light path (but in the opposite direction).
Since the fluorescent light generally consists of a broad
band of wavelengths different from the wavelength(s) of
the incident stimulating beam, and since the system

should be designed to work with a variety of fluoro-
chromes, the system including the objective 27 is
preferably largely achromatic and provides correction of
chromatic aberrations over a range of wavelengths.

5   The retro-beam 31 may pass through an aperture
or pupil of a spatial filter or stop 32 and through the
beamsplitter 23. The beamsplitter 23 may act as a
dichroic filter, reflecting the incident beam wave-
length(s) and transmitting the fluorescent wavelengths
10   (or vice versa), or can be any other type of beamsplitter
capable of separating the light of the incident and
retro-beams 19 and 31 (e.g., a polarization-sensitive
beamsplitter in combination with a quarterwave plate).
The retro-beam 31 may then be directed through a bandpass
15   filter 34 and a focusing lens 33 onto limiting aperture
35. Light passing through the aperture 35 impinges upon
a photodetector 39, such as a photomultiplier tube (PMT).
        Whichever imaging system is used, it should
preferably be capable of scanning at high speed over a
20   large scan field with high resolution imaging and minimal
optical aberrations. It should provide coaxial illumina-
tion and collection with high collection efficiency. An
achromatic system with excellent color correction, as
well as a system designed for minimizing background noise
25   (including autofluorescence) is preferred.
        With reference to Fig. 2, a sample substrate 51
of the present invention has on its upper surface 53 a
fluorescent sample 55. The substrate 51 is designed to
work with any fluorescence imaging system, like that
30   shown in Fig. 1. Such a system is shown schematically in
Fig. 2, with a laser light beam 57 providing a fluores-
cence-stimulating beam 59 of wavelength λ_1 that is
directed through an objective 61 to the sample 55 on the
surface 53. The sample 55 emits fluorescent light of
35   wavelength λ_2, which is collected by the objective 61,
separated from the incident light by a beamsplitter 63
and directed onto a PMT detector 65. The improved
substrate 51 is constructed to maximize fluorescent

emission and collection without having to increase the
power of the laser beam 59 and without having to change
the objective 61 or other optics in the system other than
the substrate 51 itself.

5   With reference to Figs. 3A-3B and 4A-4B, the
approach taken is to mirrorize the substrate onto which
the fluorescent sample 55 is deposited. From the stand-
point of geometric or "ray" optics, one might reason that
such an approach should double the excitation by the
10   incident light (because light rays would then illuminate
the sample both directly and upon reflection) and should
also double collection of the emitted fluorescence
(because the fluorescent rays emitted away from the
objective would be reflected back toward the objective,
15   thereby effectively doubling the solid angle of
collection). However, it has been discovered that this
approach by itself does not work. If a bare reflective
substrate 51' is used, as seen in Fig. 3A, then the
sample 55 is actually found to experience only negligible
20   excitation, and little, if any, fluorescence is observed,
despite the fact that the reflectivity observed in the
far field is increased as expected.
            To understand this unexpected phenomenon we
have to turn to a combination of physical optics and the
25   wave theory of light. In 1890, O. Wiener reported his
experiments showing standing waves produced by reflecting
light at normal incidence from a polished mirror. By
placing a thin photographic film at an incline to the
mirror surface and then developing the exposed film, a
30   system of separated dark bands on the film was produced.
Decreasing the inclination angle caused the bands to move
farther apart. The experiment was repeated two years
later by P. Drude and W. Nernst using fluorescence to
observe the phenomenon immediately without the need for
35   photographic development. This phenomenon is understood
to result from the superposition of incident and
reflected light waves, producing standing waves with
nodes and antinodes at different heights above the mirror

surface. There is a node located at the reflecting
surface (due to the finite conductivity of real metals,
this is actually slightly below the physical surface) and
the nodes are separated by a distance of one-half
5   wavelength.

The problem that this phenomenon creates when
attempting to produce fluorescence of a molecular sample
on a bare mirror surface is that only negligible excita-
tion occurs because the sample material is located near a
10   node in the standing wave of the excitation light. As
seen in Fig. 3A, a molecular sample 55 is located on top
of the physical surface 53' of a bare metal substrate
51'. The reflective surface is slightly below the
physical surface 53' by a distance δ corresponding to the
15   sum of the skin depth of the metal substrate 51' and the
depth of ordinary surface oxidation. In the case of an
aluminum substrate, the skin depth is about 13 nm thick
and the surface oxide is about 2 to 5.5 nm thick, for a
distance δ of about 16.5 nm. For 532 nm wavelength
20   incident light, this distance δ amounts to about 4% of a
wavelength. As seen in Fig. 3B, the field amplitude (for
light, the electric field is the primary one of interest)
is near zero at the location 63 of the molecular sample,
since it is near a node of the standing wave set up by
25   reflection. At best, the field amplitude seen by the
sample would be only about 23% of the maximum amplitude
at the location of an antinode. (The intensity, which
goes as the square of the amplitude, is only 5% of
maximum). The sample experiences little excitation,
30   despite being located above a reflective surface, and
produces very little fluorescence as a result.

Referring now to Fig. 4A, the sample substrate
51 of the present invention solves this problem by
recognizing that adding a transparent coating 53 of the
35   proper thickness onto the reflective base can create a
substrate 51 that locates a molecular sample at or near
an antinode of the standing wave of the excitation light,
thereby significantly increasing excitation and the

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resulting fluorescence emission. The coating 53 on
substrate 51 should be such that the optical path length
from the sample-coating interface to the skin depth
within the reflective base is substantially equal to
5   one-quarter wavelength.

        In order to correctly determine the proper
coating thickness, the incident angle of the excitation
light and the refractive indices of the coating material
and of the reflective base's surface oxidation must be
10   taken into account. For normal incidence, the optical
path length (OPL) is equal to the sum of the skin depth,
the effective oxidation thickness and the effective
coating thickness. For an aluminum base, the skin depth
is about 13.1 nm. The aluminum oxide is about 3.5 nm
15   thick and has a refractive index at 532 nm wavelength of
about 1.772, for an effective optical thickness of about
6.2 nm. For 532 nm laser excitation light, a quarter
wavelength is 133 nm. Thus, the coating must have an
effective optical thickness of 114 nm. If we choose a
20   silica coating 53, with a refractive index of 1.547 at
532 nm wavelength, we need a coating thickness of about
73.5 nm. If we use a different excitation source with a
different wavelength of light, or a different reflective
base or transparent coating material or a different
25   incidence angle, the coating thickness will differ but
should again be calculated to provide a one-quarter
wavelength optical thickness (or odd multiples thereof).

        As seen in Fig. 4B, when the proper coating
thickness is provided, the sample material 55 will be
30   located at or near an antinode of the standing wave of
the excitation light, i.e. near the position 61 where the
electron field amplitude (and intensity) is at a peak.
With maximum excitation, maximum fluorescence occurs.
Even if the coating thickness is not exactly correct for
35   the excitation wavelength, if the intensity is only 90%
or 95% of the peak intensity, the fluorescence signal
will still be significantly improved over prior sample
substrates. Variations from the ideal thickness can

occur due to uncertainties in skin depth, sample-to-
sample variation in metal oxidation of the base prior to
coating, and coating variations. Further, if two or more
different wavelengths are to be used with the same

5   substrate, a compromise thickness may be chosen that is
adequate for all of those wavelengths, but possibly ideal
for none. That is, the fluorescence imaging system of
Fig. 1 could have one or more light sources providing
multiple fluorescence excitation wavelengths, either

10   simultaneously or selectably, for different fluorescent
sample constituents, and the nominal optical thickness of
the sample substrate can be selected to be approximately
one-quarter wavelength (or an odd multiple) for each of
the different excitation wavelengths. Further , the

15   sample substrate could be mounted on a tiltable support
to vary the tilt angle or orientation so as to select
light incidence angle that allows the optical path to
substantially match the quarter-wavelength condition for
a selected one of the different wavelengths. When a

20   different wavelength is selected, the substrate could be
reoriented accordingly.

Base metals may include aluminum, silver, gold,
rhodium, etc. It can even be a reflective coating layer
on a glass slide or other rigid bottom layer. The

25   transparent coating may be silica (SiO_2), alumina (Al_2O_3),
magnesium fluoride (MgF_2) or some other dielectric
material. It could also be multiple layers. The layer,
or top layer, is not necessarily an inert material, but
could be biologically active so as to bind with the

30   sample material or a particular constituent of the
sample.

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Claims

1. A sample substrate adapted for use with a particular
fluorescence excitation wavelength, comprising
        a rigid base with a generally flat, smooth,
specularly reflective surface, and
        a transparent coating layer deposited on said
reflective surface of said base, said coating layer
having a thickness selected for a particular fluorescence
excitation wavelength of light such that an optical path
from the top of said coating layer to the reflecting
surface in said base is an odd multiple of one-quarter
wavelength of said light,
        whereby any fluorescent sample material
disposed on top of said coating layer would be located
near an antinode of a standing wave of said particular
fluorescence excitation light wavelength incident on said
substrate.

2. The substrate of claim 1 wherein said coating layer
thickness is selected for normal incidence of said
excitation light.

3. The substrate of claim 1 wherein said optical path is
substantially one-quarter wavelength of said light.

4. The substrate of claim 1 wherein said base is
composed of metal.

5. The substrate of claim 1 wherein said base is
composed of a rigid bottom layer with a top metal
coating.

6. The substrate of claim 1 wherein said reflective
surface is a metal selected from the group consisting of
aluminum, silver, gold and rhodium.

7. The substrate of claim 1 wherein said reflective
surface has a surface oxidation layer thereon.

8. The substrate of claim 1 wherein said coating layer
is composed of a dielectric material.

9. The substrate of claim 8 wherein said coating layer
is a dielectric selected from the group consisting of
silica, alumina and a fluoride material.

10. The substrate of claim 8 wherein said coating layer
includes multiple layers, the top layer of said multiple
layers being biologically active for binding a specified
sample constituent.

11. A fluorescence imaging system, comprising
        a sample substrate for containing a fluorescent
sample material on a top surface thereof,
        a light source providing light directed to said
sample material on said sample substrate, said light
including a fluorescence excitation wavelength particular
to a specified fluorescent constituent of said sample
material, and
        detection means for collecting and detecting
fluorescent light emitted by said sample material
containing said specified fluorescent constituent,

wherein said sample substrate includes a rigid
base with a generally flat, smooth, specular reflective
surface, and a transparent coating layer deposited on
said reflective surface of said base, said coating layer
5   having a thickness selected such that an optical path
from the top of said coating layer to the reflecting
surface in said base is an odd multiple of one-quarter of
said fluorescence excitation wavelength,
        whereby any sample material containing said
10   specified fluorescent constituent and disposed on said
top surface of said coating layer would be located near
an antinode of a standing wave of said particular
fluorescence excitation light wavelength incident on said
substrate.
15

12.   The system of claim 11 wherein said light source
provides light directed at normal incidence onto said
sample substrate.
20

13.   The system of claim 11 wherein said optical path is
substantially one-quarter wavelength of said light.

25

14.   The substrate of claim 11 wherein said base is
composed of metal.

30   15.   The substrate of claim 11 wherein said base is
composed of a rigid bottom layer with a top metal
coating.

35   16.   The substrate of claim 11 wherein said reflective
surface is a metal selected from the group consisting of
aluminum, silver, gold and rhodium.

17.   The substrate of claim 11 wherein said reflective
surface has a surface oxidation layer thereon.

5   18.   The substrate of claim 11 wherein said coating layer
is composed of a dielectric material.

19.   The substrate of claim 18 wherein said coating layer
10   is a dielectric selected from the group consisting of
silica, alumina and a fluoride material.

20.   The substrate of claim 18 wherein said coating layer
15   includes multiple layers, the top layer of said multiple
layers being chemically reactive for binding a specified
biological sample constituent.

20   21.   A method of imaging a sample material containing a
specified fluorescent constituent, comprising
          disposing a sample material on a sample
substrate,
          directing light including a fluorescence
25   excitation wavelength particular to a specified
fluorescent constituent into said sample material, said
light thereby stimulating fluorescent emission by said
sample material whenever said specified fluorescent
constituent is present, and
30   collecting and detecting any fluorescent light
emitted from said sample material,
          wherein said sample substrate includes a rigid
base with a generally flat, smooth, specular reflective
surface, and a transparent coating layer deposited on
35   said reflective surface of said base, said coating layer
having a thickness selected such that an optical path
from the top of said coating layer to the reflecting

surface in said base is an odd multiple of one-quarter of
said fluorescence excitation wavelength,

whereby any sample material containing said
specified fluorescent constituent and disposed on said

5   top surface of said coating layer would be located near
an antinode of a standing wave of said particular
fluorescence excitation light wavelength incident on said
substrate.

10

22. A fluorescence imaging system, comprising
a sample substrate for containing a fluorescent
sample material on a top surface thereof,
at least one light source providing light

15   directed to said sample material on said sample
substrate, said light from each said light source
including a fluorescence excitation wavelength particular
to a specified fluorescent constituent of said sample
material, such that different wavelengths of light can

20   excite fluorescence from different constituents in said
sample material, and
detection means for collecting and detecting
fluorescent light emitted by said sample material
containing a plurality of different fluorescent

25   constituents of said sample material,
wherein said sample substrate includes a rigid
base with a generally smooth, specular reflective
surface, and a transparent coating layer deposited on
said reflective surface of said base, said coating layer

30   having a thickness selected such that an optical path
from the top of said coating layer to the reflecting
surface of said base is approximately an odd multiple of
one-quarter wavelength for light of each fluorescence
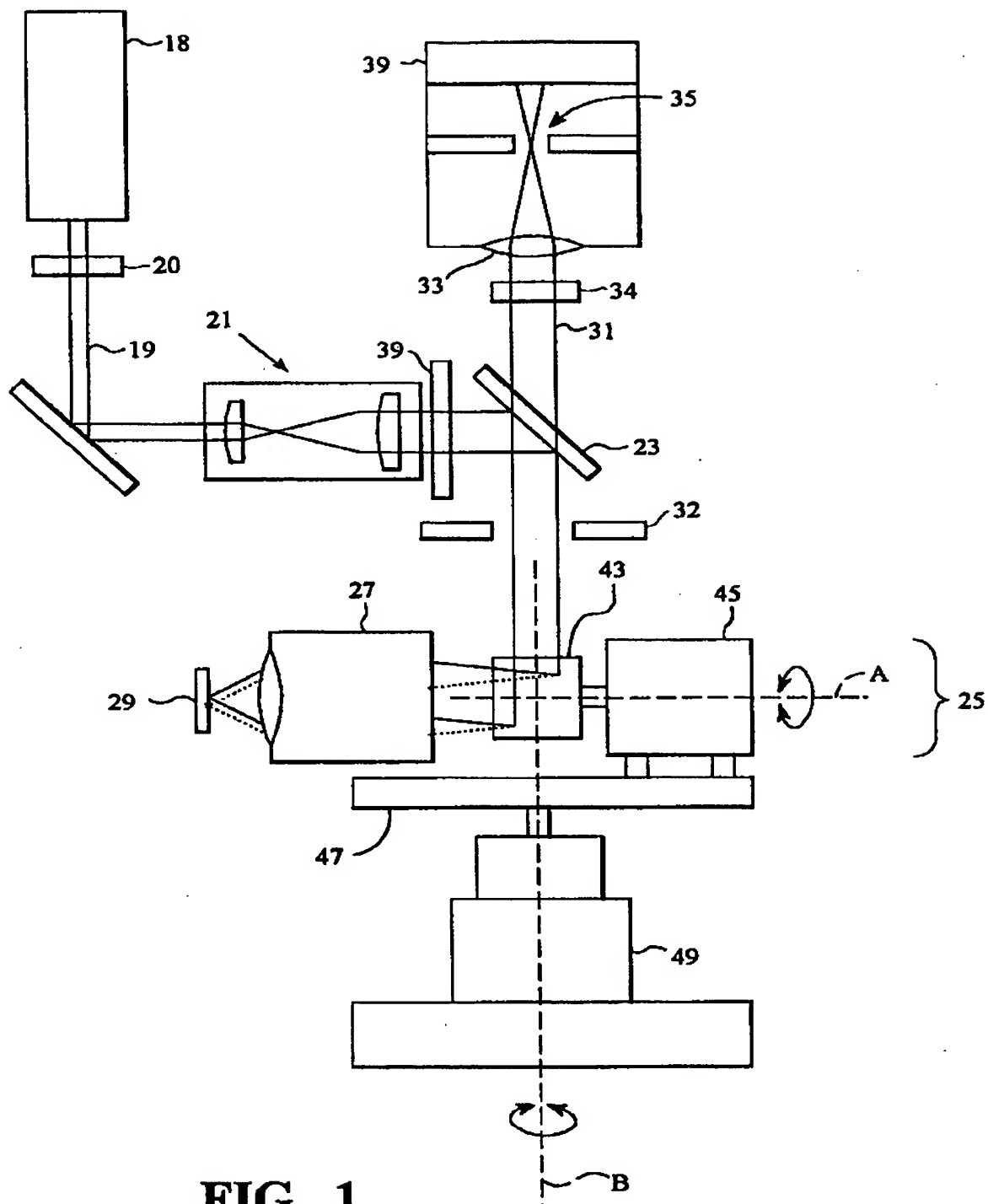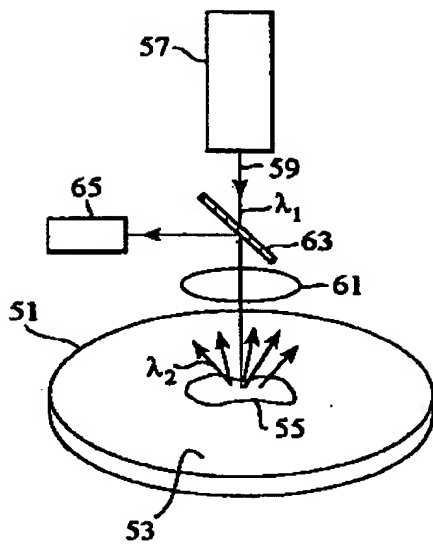excitation wavelength from said at least one light

35   source.

23. The system of claim 22 further including sample
substrate support and excitation means for tilting said
sample substrate to vary an angle of incidence of light
from said at least one light source, such that for each
5   selected fluorescence excitation wavelength, a tilt angle
of said substrate is also selected so that said optical
path is substantially equal to an odd multiple of one-
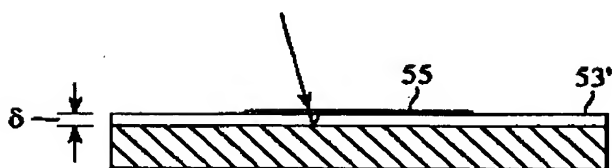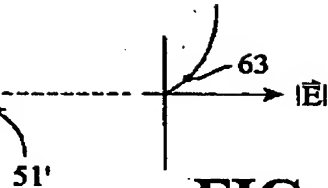quarter of that selected wavelength.

10
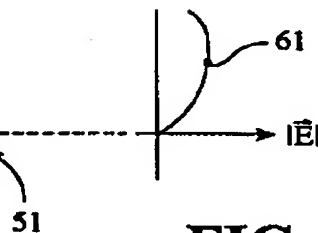
FIG. 1

FIG. 2

FIG. 3A

FIG. 3B

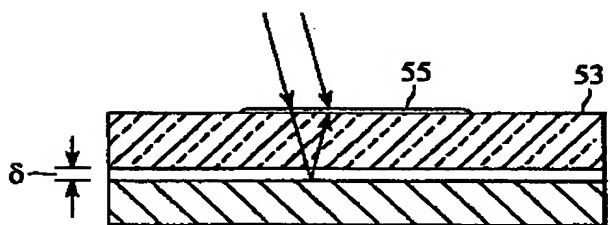
FIG. 4A

FIG. 4B

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6)   :G01N 21/64
US CL   :250/458.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. :   250/458.1, 461.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS antinode(p)fluorescen?

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,394,268 A (LANNI et al.) 28 FEBRUARY 1995 (28.02.95), col. 5, lines 51-54 and 58-63.	1, 2, 4-8, 11, 12, 14-18, 21, 22
A	US 3,591,805 A (SCHOEFFEL) 6 JULY 1971 (06.07.71).	1, 8-10
A	DE 43 01 005 A1 (DIAGEN INSTITUT FÜR MOLEKULARBIOLOGISCHE DIAGNOSTIK GmbH) 21 JULY 1994 (21.07.94).	1, 8-10

☐ Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"B"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 MARCH 1998	2 2 APR 1998

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